Conformationally Constrained Parathyroid Hormones With α-Helix Stabilizers

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Background of the Invention

Field of the Invention

The present invention relates to conformationally constrained parathyroid hormone (PTH) analogs, and methods of preparing and using the PTH analogs.

Background Art

Parathyroid hormone

Parathyroid hormone (PTH), an 84 amino acid peptide, is the principal regulator of ionized blood calcium in the human body (Kronenberg, H.M., *et al.*, In *Handbook of Experimental Pharmacology*, Mundy, G.R., and Martin, T.J., (eds), pp. 185-201, Springer-Verlag, Heidelberg (1993)). Regulation of calcium concentration is necessary for the normal function of the gastrointestinal, skeletal, neurologic, neuromuscular, and cardiovascular systems. PTH synthesis and release are controlled principally by the serum calcium level; a low level stimulates and a high level suppresses both hormone synthesis and release. PTH, in turn, maintains the serum calcium level by directly or indirectly promoting calcium entry into the blood at three sites of calcium exchange: gut, bone, and kidney. PTH contributes to net gastrointestinal absorption of calcium by favoring

the renal synthesis of the active form of vitamin D. PTH promotes calcium resorption from bone indirectly by stimulating differentiation of the bone-resorbing cells, osteoclasts. It also mediates at least three main effects on the kidney: stimulation of tubular calcium reabsorption, enhancement of phosphate clearance, and promotion of an increase in the enzyme that completes synthesis of the active form of vitamin D. PTH is thought to exert these effects primarily through receptor-mediated activation of adenylate cyclase and/or phospholipase C.

Disruption of calcium homeostasis may produce many clinical disorders (*e.g.*, severe bone disease, anemia, renal impairment, ulcers, myopathy, and neuropathy) and usually results from conditions that produce an alteration in the level of parathyroid hormone. Hypercalcemia is a condition that is characterized by an elevation in the serum calcium level. It is often associated with primary hyperparathyroidism in which an excess of PTH production occurs as a result of a parathyroid gland lesion (*e.g.*, adenoma, hyperplasia, or carcinoma). Another type of hypercalcemia, humoral hypercalcemia of malignancy (HHM) is the most common paraneoplastic syndrome. It appears to result in most instances from the production by tumors (*e.g.*, squamous, renal, ovarian, or bladder carcinomas) of a class of protein hormone which shares amino acid homology with PTH. These PTH-related proteins (PTHrP) appear to mimic certain of the renal and skeletal actions of PTH and are believed to interact with the PTH receptor in these tissues.

Osteoporosis

Osteoporosis is a potentially crippling skeletal disease observed in a substantial portion of the senior adult population, in pregnant women and even in juveniles. The term osteoporosis refers to a heterogeneous group of disorders. Clinically, osteoporosis is separated into type I and type II. Type I osteoporosis occurs predominantly in middle aged women and is associated with estrogen loss at menopause, while osteoporosis type II is associated with advancing age. Patients with osteoporosis would benefit from new therapies designed to promote

fracture repair, or from therapies designed to prevent or lessen the fractures associated with the disease.

The disease is marked by diminished bone mass, decreased bone mineral density (BMD), decreased bone strength and an increased risk of bone fracture. At present, there is no effective cure for osteoporosis, though estrogen, calcitonin and the bisphosphonates, etidronate and alendronate are used to treat the disease with varying levels of success. These agents act to decrease bone resorption. Since parathyroid hormone regulates blood calcium and the phosphate levels, and has potent anabolic (bone-forming) effects on the skeleton, in animals (Shen, V., *et al., Calcif. Tissue Int. 50*:214-220 (1992); Whitefild, J.F., *et al., Calcif. Tissue Int. 56*:227-231 (1995) and Whitfield, J.F., *et al., Calcif. Tissue Int. 60*:26-29 (1997)) and humans (Slovik, D.M., *et al., J. Bone Miner. Res. 1*:377-381 (1986); Dempster, D.W., *et al., Endocr. Rev. 14*:690-709 (1993) and Dempster, D.W., *et al., Endocr. Rev. 15*:261 (1994)) when administered intermittently, PTH, or PTH derivatives, are prime candidates for new and effective therapies for osteoporosis.

PTH Derivatives

PTH derivatives include polypeptides that have amino acid substitutions or are truncated relative to the full length molecule. Both a 14 and a 34 amino acid amino-terminal truncated form of PTH, as well as a C-terminal truncated form have been studied. Additionally, amino acid substitutions within the truncated polypeptides have also been investigated.

Synthetic PTH(1-34) exhibits full bioactivity in most cell-based assay systems, has potent anabolic effects on bone mass in animals and has been shown to reduce the risk of bone fracture in postmenopausal osteoporotic women (Neer, R.M., *et al., N.E.J.M. 344*:1434-1441 (2001); Dempster, D.W., *et al., Endocr Rev 14*:690-709 (1993)). PTH acts on the PTH/PTHrP receptor (P1R), a class II G protein-coupled heptahelical receptor that couples to the adenylyl cyclase/cAMP and phospolipase C/inositol phosphate (IP) signaling pathway (Rippner, H., *et al., Science 254*:1024-1026 (1991)). Deletion analysis studies have shown that the amino-terminal residues of PTH play a crucial role in stimulating the P1R to

activate the cAMP and IP signaling pathways (Tregear, G.W., *et al., Endocrinology 93*:1349-1353 (1973); Takasu, H., *et al., Biochemistry 38*:13453-13460 (1999)). Crosslinking and receptor mutagenesis studies have indicated that residues in the amino-terminal portion of PTH interact with the extracellular loops and extracellular ends of the seven transmembrane helices, which reside within the juxtamembrane region of the receptor (Bergwitz, C., *et al., J. Biol. Chem. 271*:26469-26472 (1996); Hoare, S.R.J., *et al., J. Biol. Chem 276*:7741-7753 (2001); Behar, V., *et al., J. Biol. Chem. 275*:9-17 (1999); Shimizu, M., *et al., J. Biol. Chem. 275*:19456-19460 (2000); Luck, M.D., *et al., Molecular Endocrinology 13*:670-680 (1999)).

α-Helix Stabilizers

The first 34 amino acids of PTH and PTHrP contain sufficient information for high affinity P1R binding and potent induction of P1R-mediated signaling responses (Neer, RM, *et al., N.E.J.M. 344*: 1434-1441(2001)). Short N-terminal fragments of PTH, such as PTH(1-14) and PTH(1-11) exhibit extremely weak binding affinities (Kd>>100μM) but are nonetheless capable of eliciting cAMP-signaling responses, albeit with potencies (EC50s≥100μM) that are substantially weaker than that of PTH(1-34)(EC50~2nM)(Luck, *MD et al., Molecular Endocrinology 13*: 670-680(1999)). It has been reported that a series of modified PTH(1-14) and PTH(1-11) analogs exhibit signaling potencies that are nearly, or even fully, equal to that of PTH(1-34)(Shimizu, M *et al., Endocrinology 142*: 3068-3074(2001); Shimizu, M. *et al., J. Biol. Chem. 276*: 490003-49012(2001); Shimizu, M. *et al., J.Biol.Chem. 275*: 21836-21843(2000)).

Recently, it was also reported that PTH(1-14) analogs containing the α,α-disubstitued amino acid, α-aminoisobutyric acid (Aib) at positions 1 and/or 3, have 10- to 100- fold higher affinities and cAMP signaling potencies than do their counterpart peptides containing alanine at these positions (Shimizu, N. *et al. J. Biol. Chem. 276*: 49003-49012 (2001)).

Brief Summary of the Invention

The invention provides novel PTH polypeptide derivatives containing amino acid substitutions at selected positions in the polypeptides. The derivatives function as full, or nearly full, agonists of the PTH-1 receptor. Because of their unique properties, these polypeptides have utility as drugs for treating human diseases of the skeleton, such as osteoporosis.

The invention provides derivatives of PTH(1-14), PTH(1-13), PTH(1-12), PTH(1-11), PTH(1-10) and PTH (1-9) polypeptides, wherein at least one residue in each polypeptide is an α,α-di-substituted amino acid. These polypeptides may also contain a residue which is a helix, preferably an α-helix, stabilizing residue. These α-helix stabilizing residues include, but are not limited to α,α-di-alkyl amino acids with structurally varied sidechains, such as: α-aminoisobutyric acid (Aib), α,α-diethyl-glycine (Deg), 1-aminocyclopropane-1-carboxylic acid (Ac_3c), 1-aminocyclopentane-1-carboxylic acid (Ac_5c), amino-cyclobutane-1-carboxylic acid (Ac_4c), and 1-amino-cyclohexane-1-carboxylic acid (Ac_6c). The invention also provides methods of making such peptides. Further, the invention encompasses compositions and methods for use in limiting undesired bone loss in a vertebrate at risk of such bone loss, in treating conditions that are characterized by undesired bone loss or by the need for bone growth, e.g. in treating fractures or cartilage disorders and for raising cAMP levels in cells where deemed necessary.

In one aspect, the invention is directed to a biologically active peptide consisting essentially of X_{01}ValX_{02}GluIleGlnLeuMetHis$X_{03}X_{04}X_{05}X_{06}X_{07}$(SEQ. ID. NO.1), wherein X_{01} is an α-helix-stabilizing residue, Gly, Ser or Ala; X_{02} is an α-helix-stabilizing residue, Ala or Ser; X_{03} is Ala, Gln or Asn; X_{04} is Arg, Har or Leu; X_{05} is an α-helix stabilizing residue, Ala or Gly; X_{06} is an α-helix stabilizing residue or Lys; X_{07} is an α-helix stabilizing residue, Trp or His; wherein at least one of X_{01}, X_{02}, X_{03}, X_{04}, X_{05}, X_{06} or X_{07} is an α-helix stabilizing residue, and wherein at least one of said α-helix stabilizing residues is Aib (α-aminoisobutyric acid), Ac_3c (1-aminocyclopropane-1-carboxylic acid), Ac_4c (1-

amino-cyclobutane-1-carboxylic acid), Ac_5c (1-aminocyclopentane-1-carboxylic acid), Ac_6c(1-amino-cyclohexane-1-carboxylic acid), or Deg (α,α-diethylglycine).

The invention is further drawn to fragments of the peptide having the sequence of SEQ. ID. NO. 1, in particular X_{01}ValX_{02}GluIleGlnLeuMetHis$X_{03}X_{04}X_{05}X_{06}$ (part of SEQ. ID. NO. 1), X_{01}ValX_{02}GluIleGlnLeuMetHis$X_{03}X_{04}X_{05}$ (part of SEQ. ID. NO. 1), X_{01}ValX_{02}GluIleGlnLeuMetHis$X_{03}X_{04}$ (part of SEQ. ID. NO. 1), X_{01}ValX_{02}GluIleGlnLeuMetHisX_{03} (part of SEQ. ID. NO. 1), and X_{01}ValX_{02}GluIleGlnLeuMetHis (part of SEQ. ID. NO. 1). The invention further encompasses pharmaceutically acceptable salts of the above-described peptides, and N- or C-derivatives of the peptides, wherein at least one of X_{01}, X_{02}, $X_{03,}X_{04}$, X_{05}, or X_{06} is an α-helix-stabilizing residue. One or more of the α-helix-stabilizing residue may be selected from the group consisting of Aib, Ac_3c, Ac_4c, Ac_5c, Ac_6c, or Deg.

In addition, the invention is drawn to a biologically active polypeptide consisting essentially of X_{01}ValX_{02}GluIleGlnLeuMetHisGlnHarAlaLysTrp (SEQ. ID. NO. 2) as well as fragment peptides containing amino acids 1-13, 1-12, 1-11, 1-10, or 1-9. The invention further encompasses pharmaceutically acceptable salts of the above-described peptides and N- or C-derivatives of the peptides, wherein X_{01} is an α-helix-stabilizing residue, Gly, Ser or Ala; and X_{02} is an α-helix-stabilizing residue, Ala, or Ser. α-helix stabilizing residues include, but are not limited to, the group consisting of Aib, Ac_3c, Ac_4c, Ac_5c, Ac_6c, and Deg as defined above.

Preferred embodiments of the biologically active peptide include: Ac_5cValAibGluIleGlnLeuMetHisGlnArgAlaLysTrpNH_2 (SEQ.ID.NO. 30), Ac_5cValAlaGluIleGlnLeuMetHisGlnHarAlaLysTrpNH_2 (SEQ. ID. NO. 4), AibValAc_5cGluIleGlnLeuMetHisAsnLeuGlyLysHisNH_2(SEQ. ID. NO. 32), and Ac_5cValAibGluIleGlnLeuMetHisNH_2 (SEQ. ID. NO. 31). It is contemplated that fragments of the above mentioned peptides, containing amino acids 1-9, 1-10, 1-11, 1-12 or 1-13, are also embodiments of the present invention. The invention

further encompasses pharmaceutically acceptable salts of the above-described peptides, and N- or C-derivatives of the peptides.

In another aspect, the invention is directed to a biologically active peptide consisting essentially of X_{01}ValX_{02}GluIleX_{03}LeuMetHis$X_{04}X_{05}X_{06}$LysX_{07} (SEQ. ID. NO. 5), wherein X_{01} is an α-helix-stabilizing residue, Gly, Ser or Ala; X_{02} is an α-helix-stabilizing residue, Ala or Ser; X_{03} is Ala, Gln or Asn; X_{04} is Ala, Gln or Asn; X_{05} is an α-helix-stabilizing residue, Ala, Gly, Har or Arg; X_{06} is an α-helix-stabilizing residue or Lys; and X_{07} is an α-helix-stabilizing residue, Trp or His; wherein at least one of X_{01}, X_{02}, X_{03}, X_{04}, X_{05}, X_{06} or X_{07} is an α-helix-stabilizing residue, and wherein at least one of said α-helix stabilizing residues is Aib, Ac_3c, Ac_4c, Ac_5c, Ac_6c, or Deg.

The invention is further drawn to fragments of the peptide having the sequence of SEQ. ID. NO. 5, in particular X_{01}ValX_{02}GluIleX_{03}LeuMetHis$X_{04}X_{05}X_{06}$Lys (part of SEQ. ID. NO. 5), X_{01}ValX_{02}GluIleX_{03}LeuMetHis$X_{04}X_{05}X_{06}$ (part of SEQ. ID. NO. 5), X_{01}ValX_{02}GluIleX_{03}LeuMetHis$X_{04}X_{05}$ (part of SEQ. ID. NO. 5), X_{01}ValX_{02}GluIleX_{03}LeuMetHisX_{04} (part of SEQ. ID. NO. 5), and X_{01}ValX_{02}GluIleX_{03}LeuMetHis (part of SEQ. ID. NO. 5), wherein X_{01} is an α-helix-stabilizing residue, Gly, Ser or Ala; X_{02} is an α-helix-stabilizing residue, Ala, or Ser; X_{03} is Ala, Gln or Asn; X_{04} is Ala, Gln or Asn; X_{05} is an α-helix-stabilizing residue, Ala , Gly, Har or Arg; X_{06} is an α-helix-stabilizing residue or Lys; and X_{07} is an α-helix-stabilizing residue, Trp or His; wherein at least one of X_{01}, X_{02}, X_{03}, X_{04}, X_{05}, X_{06} or X_{07} is an α-helix-stabilizing residue, and wherein at least one of said α-helix stabilizing residues is Aib, Ac_3c, Ac_4c, Ac_5c, Ac_6c, or Deg. The invention further encompasses pharmaceutically acceptable salts of the above-described peptides, and N- or C- derivatives of the peptides.

In addition, the invention is drawn to a biologically active polypeptide consisting essentially of X_{01}ValX_{02}GluIleGlnLeuMetHisGlnHarAlaLysTrp-amide (SEQ. ID. NO. 6) and fragment peptides containing amino acids 1-13, 1-12, 1-11, 1-10, or 1-9. The invention further encompasses pharmaceutically acceptable salts of the above-described peptides, and N- or C-derivatives of the peptides, wherein

X_{01} is an α-helix-stabilizing residue, Gly, Ser or Ala; and X_{02} is an α-helix-stabilizing residue, Ala or Ser, and wherein at least one of said α-helix stabilizing residues is Aib, Ac_3c, Ac_4c, Ac_5c, Ac_6c, or Deg.

Preferred embodiments of the biologically active peptide include: Ac_4cValAibGluIleGlnLeuMetHisGlnHarAlaLysTrp (SEQ. ID. NO. 7), Ac_6cValAibGluIleGlnLeuMetHisGlnHarAlaLysTrp (SEQ. ID. NO. 8), Ac_5cVal Ac_4cGluIleGlnLeuMetHisGlnHarAlaLysTrp (SEQ. ID. NO. 9), Ac_5cValAc_6cGluIleGlnLeuMetHisGlnHarAlaLysTrp (SEQ. ID. NO. 10), Ac_4cVal Ac_4cGluIleGlnLeuMetHisGlnHarAlaLysTrp (SEQ. ID. NO. 11), and Ac_6cVal Ac_6cGluIleGlnLeuMetHisGlnHarAlaLysTrp (SEQ. ID. NO. 12). It is contemplated that fragments of the above mentioned peptides, containing amino acids 1-9, 1-10, 1-11, 1-12, or 1-13, are also embodiments of the present invention. The invention further encompasses pharmaceutically acceptable salts of the above-described peptides, and N- or C- derivatives of the peptides.

This invention also provides pharmaceutical compositions comprising any of the PTH derivatives described herein and a pharmaceutically acceptable excipient and/or a pharmaceutically acceptable solution such as saline or a physiologically buffered solution.

In one aspect, the invention is directed to a pharmaceutical composition comprising the biologically active peptide having the sequence of SEQ. ID. NOs. 1, 5 or any of the above peptides, and a pharmaceutically acceptable carrier.

In another aspect, the invention is directed to a method of making SEQ. ID: NO. 1, 5 or any of the above peptides, wherein the peptide is synthesized by solid phase synthesis, liquid phase synthesis, or solution phase synthesis.

In another aspect, the invention is directed to a method of making SEQ. ID. NO. 1,5 or any of the above peptides, wherein the peptide is protected by FMOC.

This invention also provides a method for treating mammalian conditions characterized by decreases in bone mass, the method of which comprises administering to a subject in need thereof an effective bone mass-increasing amount of a biologically active PTH polypeptide derivative. A preferable embodiment of the invention is drawn to conditions such as osteoporosis,

hyperparathyroidism and hypercalcemia. The types of osteoporosis include, but are not limited to old age osteoporosis and postmenopausal osteoporosis.

In another aspect, the invention is directed to a method for treating mammalian conditions characterized by decreases in bone mass, the method comprising administering to a subject in need thereof an effective bone mass-increasing amount of a biologically active peptide of having the sequence of SEQ. ID. NO. 1 or 5, or any of the above peptides and a pharmaceutically acceptable carrier. Additional preferable embodiments include using an effective amounts of the polypeptide of about 0.01 μg/kg/day to about 1.0 μg/kg/day wherein the polypeptide is administered parenterally, subcutaneously or by nasal insufflation.

This invention also provides a method for determining rates of bone reformation, bone resorption and/or bone remodeling comprising administering to a patient an effective amount of a labeled PTH polypeptide, such as for example, a peptide having the sequence of SEQ. ID. NO. 1, 5 or derivatives thereof and determining the uptake of the peptide into the bone of the patient. The peptide may be labeled with a label selected from the group consisting of: radiolabels, flourescent labels, bioluminescent labels, or chemiluminescent labels. In a preferable embodiment the radiolabel is ^{125}I or ^{99m}Tc.

Brief Description of the Figures

FIG. 1. α,α- disubstituted amino acid analog structures Aib, Deg, Ac_3c, Ac_5c. Note the restricted φ/ψ rotation favors helix formation. (modified from R.Kau. and P. Balaram, *Bioorganic Medicinal Chemistry*, 7 105-117(1999)).

FIG. 2. Effect of single α,α-substituted amino acid substitutions at position 1 or 3 in a PTH(1-14) analog on cAMP-stimulating potency and P1R-binding affinity in HKRK-B28 cells. The peptide [$Ala^{1,3,12}$, Gln^{10}, Har^{11}, Trp^{14}]PTH(1-14)NH_2(parent)(SEQ. ID. NO. 13) and analogs thereof in which alanine-1 or alanine-3 were substituted with 1-aminocyclopropane-1-carboxylic acid (Ac_3c); 1-aminocyclopentane-1-carboxylic acid (Ac_5c) or α,α-diethylglycine (Deg) were evaluated in HKRK-B28 cells for their capacity to stimulate intracellular cAMP accumulation (A), (C) and the capacity to inhibit binding of ^{125}I-

[$Aib^{1,3}$,Nle^{8},Gln^{10},Har^{11},Ala^{12}, Trp^{14},Arg^{19},Tyr^{21}]rPTH(1-21)NH_2 (SEQ. ID. NO. 36) (B), (D). The cAMP responses are expressed as a percent of the maximum response observed in each experiment for the parent peptide, the average of which was 225$\pm$21 picomoles of cAMP per well (n=7); the corresponding basal cAMP level was 3.8$\pm$0.1 picomoles per well. Peptides and their corresponding symbols are identified in the key.

FIG. 3. Effects of combined α,α-disubstituted amino acid substitutions at positions 1 and 3 in a PTH(1-14) analog on cAMP-stimulating potency and binding affinity in HKRK-B28 cells. The peptide [Ac_5c^{1},Aib^{3},Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2 (SEQ. ID. NO. 15) and [$Deg^{1,3}$,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2 (SEQ. ID. NO. 27) were evaluated in HKRK-B28 cells for the capacity to stimulate intracellular cAMP accumulation (A) and the capacity to inhibit the binding of ^{125}I-[$Aib^{1,3}$,Nle^{8},Gln^{10},Har^{11},Ala^{12}, Trp^{14},Arg^{19},Tyr^{21}]rPTH(1-21)NH_2 (SEQ. ID. NO. 36) (B). Also shown is the response observed with the parent peptide [$Ala^{1,3,12}$,Gln^{10},Har^{11},Trp^{14}]PTH(1-14)NH_2 (SEQ. ID. NO. 13). The cAMP responses are expressed as a percent of the maximum response observed in each experiment for the parent peptide. Peptides and corresponding symbols are identified in the key.

FIG. 4. Phospholipase-C signaling properties of PTH(1-14) analogs in COS-7 cells expressing the hP1R.The capacities of [Ac_5c^{1},Aib^{3},Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2 (SEQ. ID. NO. 15), [$Aib^{1,3}$,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2 (SEQ. ID. NO. 3), and [$Deg^{1,3}$,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2 (SEQ. ID. NO. 27) To stimulate formation of 3H-inositol phosphates (IP^1+IP^2+IP^3) in COS-7 cells transiently transfected with the hP1R, were evaluated. Peptides and corresponding symbols are defined in the key.

FIG. 5. Stimulation of cAMP formation by PTH(1-9) and PTH(1-10) analogs in HKRK-B28 cells. Panel A shows the capacity of varying concentrations of [Ac_5c^{1},Aib^{3},Gln^{10}]PTH(1-10)NH_2 (SEQ. ID. 22) to stimulate intracellular cAMP accumulation in HKRK-B28 cells. The response to [$Ala^{1,3,12}$,Gln^{10},Har^{11},Trp^{14}]PTH(1-14)NH_2 (SEQ. ID. NO. 13) is reshown from

Figure 2A. Panel B shows the cAMP levels in cells treated with buffer alone (basal) or buffer containing either native PTH(1-9)NH_2, [$Aib^{1,3}$]PTH(1-9)NH_2 (SEQ. ID. NO. 37) or [Ac_5c^1,Aib^3]PTH(1-9)NH_2 (SEQ. ID. NO. 34), each at a concentration of 100 μM (*P* versus basal =0.052(*) or <0.0001(a); *P* versus [$Aib^{1,3}$]PTH(1-9)NH_2 = 0.001(b)(SEQ. ID. NO. 37). Also analyzed were [Ac_5c^1,Aib^3,Gln^{10}]PTH(1-10)NH_2 (SEQ. ID. NO. 22) at 10 μM, and [Ac_5c^1,Aib^3,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2 (SEQ. ID.15) at 10 nM, for which 33-fold (*P*=0.0003) and 38-fold (*P*=0.006) increases in cAMP levels, relative to basal, were observed, respectively. Peptides and corresponding symbols are identified in the key.

FIG. 6. cAMP-stimulating capacities of PTH(1-14) and a PTH(1-10) analogs in LdelNt-2 cells. The peptides [$Aib^{1,3}$,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2(SEQ.ID.NO.3), [Ac_5c^1,Aib^3,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2(SEQ.ID.NO.15), [$Deg^{1,3}$,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2(SEQ. ID. NO. 27) and [Ac_5c^1,Aib^3,Gln^{10}]PTH(1-10)NH_2 (SEQ. ID. NO. 22) were evaluated for cAMP-stimulating potency in LdelNt-2 cells, which are clonal, LLC-PK1-derived cells that express hP1R-delNt via stable transfection. The data are expressed as a percent of the maximum response observed in each experiment for [Ac_5c^1,Aib^3,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2(SEQ. ID. NO.15), which was 125 ± 19 picomoles of cAMP per well; the basal cAMP levels were 1.8±0.3 picomoles per well. The corresponding EC_{50} values were 6.4±1.6nM ([$Aib^{1,3}$,Gln^{10},Har^{11},Ala^{12},Trp_{14}]PTH(1-14)NH_2)(SEQ. ID. NO. 3); 6.4±2.3nM([Ac_5c^1,Aib^3,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2(SEQ. ID. NO. 15); 1.4±0.5 μM ($Deg^{1,3}$,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2) (SEQ. ID. NO. 27) and 37±22 μM([Ac_5c^1,Aib^3,Gln^{10}]PTH(1-10)NH_2) (SEQ. ID. NO. 22). Peptides and corresponding symbols are identified in the key.

FIG. 7. Stimulation of PLC activity. The PTH(1-14) analog stimulated PLC activity in COS-7 cells transiently expressing hP1R-WT. Peptides and corresponding symbols are identified in the key.

FIG. 8. Agonist activity of PTH analogs *in vivo*. Mice were injected intra-peritoneally with vehicle or vehicle containing either [$Nle^{8,21}$,Tyr^{34}]ratPTH(1-

34)NH_2 (SEQ. ID. NO. 14), or [Ac_5c^1,Aib^3,Gln^{10},Har^{11},Ala^{12},Trp^{14}]humanPTH(1-14)NH_2 (SEQ. ID. NO. 15) and 3 minutes later blood samples were withdrawn and plasma cAMP levels were determined by radioimmunoassay. The effective PTH peptide concentrations were 20×10^{-9} mole per kilogram of body-weight for PTH(1-34) analog and 200×10^{-9} mole per kilogram of body-weight for PTH(1-14) analog. Each bar represents the mean (±s.e.m.) cAMP value of data obtained from 4 mice.

Detailed Description of the Invention

Definitions

Amino Acid Sequences: The amino acid sequences in this application use either the single letter or three letter designations for the amino acids. These designations are well known to one of skill in the art and can be found in numerous readily available references, such as for example in *Cooper, G.M., The Cell* 1997, ASM Press, Washington, D.C. or Ausubel et al., *Current Protocols in Molecular Biology,* 1994 . Where substitutions in a sequence are referred to, for example, as Ser-3 -->Ala or [Ala^3]peptide, this means that the serine in the third position from the N-terminal end of the polypeptide is replaced with another amino acid, Alanine in this instance.

Scaffold peptide: [M]PTH(1-14) is defined as [$Ala^{1,3,12}$,Gln^{10},Har^{11},Trp^{14}]PTH(1-14)amide (SEQ. ID. NO. 13). [M]PTH(1-11) is defined as [$Ala^{1,3}$,Gln^{10},Har^{11}]PTH(1-11)NH_2 (SEQ. ID. NO. 16). [$Aib^{1,3}$,M]PTH(1-14) is defined as [$Aib^{1,3}$,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2 (SEQ. ID. NO. 3).

α,α-dialkyl amino acids: "Aib" refers to α-aminoisobutyric acid; "Har" refers to homoarginine; "Nle" refers to norleucine; Ac_3c refers to 1-aminocyclopropane-1-carboxylic acid; Ac_4c refers to 1-amino-cyclobutane-carboxylic acid; Ac_5c refers to 1-aminocyclopentane-1-carboxylic acid; Ac_6c refers to 1-amino-cyclohexane-carboxylic acid; Deg refers to α,α-diethylglycine; IBMX refers to 3-isobutyl-1-methylxanthine; and other amino acids are in either the

conventional one- or three-letter codes.

Biological Activity of the Protein: This expression refers to any biological activity of the polypeptide. Examples of these activities include, but are not limited to metabolic or physiologic function of compounds of a peptide having the sequence of SEQ. ID. NO. 1 or derivatives thereof, including similar activities or improved activities, or those activities with decreased undesirable side-effects. Also included are antigenic and immunogenic activities of the above-described compounds.

Derivative or Functional Derivative: The term "derivative" or "functional derivative" is intended to include "variants," the "derivatives," or "chemical derivatives" of the PTH molecule. A "variant" of a molecule such as for example, a compound of a peptide having the sequence of SEQ. ID. NO. 1 or derivative thereof is meant to refer to a molecule substantially similar to either the entire molecule, or a fragment thereof. An "analog" of a molecule such as for example, a compound having the sequence of SEQ. ID. NO. 1 or derivative thereof is meant to refer to a non-natural molecule substantially similar to either the peptide having the sequence of SEQ. ID. NO. 1 molecules or fragments thereof.

PTH derivatives contain changes in the polypeptide relative to the native PTH polypeptide of the same size. The sequence of the native human ("hPTH") PTH (1-14) polypeptide is SerValSerGluIleGlnLeuMetHisAsnLeuGlyLysHis (part of SEQ. ID. NO. 26), or native rat ("rPTH") PTH (1-14) is AlaValSerGluIleGlnLeuMetHisAsnLeuGly LysHis (SEQ. ID. NO. 17). A molecule is said to be "substantially similar" to another molecule if the sequence of amino acids in both molecules is substantially the same, and if both molecules possess a similar biological activity. Thus, two molecules that possess a similar activity, may be considered variants, derivatives, or analogs as that term is used herein even if one of the molecules contains additional amino acid residues not found in the other, or if the sequence of amino acid residues is not identical. PTH derivatives, however, need not have substantially similar biological activity to the native molecule. In some instances PTH derivatives have substantially different activity than the native PTH. For

example, a derivative may be either an antagonist or an agonist of the PTH receptor.

As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties may improve the molecule's solubility, absorption, biological half-life, etc. The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, etc. Examples of moieties capable of mediating such effects are disclosed in *Remington's Pharmaceutical Sciences* (1980) and will be apparent to those of ordinary skill in the art.

Fragment: A "fragment" of a molecule such as for example, X_{01}ValX$_{02}$GluIleGlnLeuMetHis$X_{03}X_{04}X_{05}X_{06}$ (1-13) (part of SEQ. ID. NO. 1) or derivative thereof is meant to refer to any polypeptide subset of these molecules, including N- or C- derivatives thereof.

Fusion protein: As used herein, a "fusion protein" is a protein comprising compounds such as for example, X_{01}ValX$_{02}$GluIleGlnLeuMetHis$X_{03}X_{04}X_{05}X_{06}X_{07}$ (SEQ. ID. NO. 1), or derivatives thereof, either with or without a "selective cleavage site" linked at its N-terminus, which is in turn linked to an additional amino acid leader polypeptide sequence.

Polypeptide: Polypeptide and peptide are used interchangeably. The term polypeptide refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds, *i.e.*, peptide isosteres. "Polypeptide" refers to both short chains, commonly referred to as peptides, oligopeptides or oligomers, and to longer chains, generally referred to as proteins. Polypeptides may contain amino acids other than the 20 gene-encoded amino acids and include amino acid sequences modified either by natural processes, such as post-translational processing, or by chemical modification techniques which are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in the research literature. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini.

It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications.

Polypeptides may be branched and they may be cyclic, with or without branching. Cyclic, branched and branched cyclic polypeptides may result from post-translational modifications or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. *See*, for instance, *Proteins-Structure and Molecular Properties*, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York, 1993 and Wold, F., Posttranslational Protein Modifications: Perspectives and Prospects, pgs. 1-12 in *Posttranslational Covalent Modification of Proteins*, B. C. Johnson, Ed., Academic Press, New York, 1983; Seifter *et al.*, "Analysis for protein modifications and nonprotein cofactors", *Methods in Enzymol. 182*:626-646 (1990) and Rattan *et al.*, "Protein Synthesis: Posttranslational Modifications and Aging", *Ann NY Acad Sci 663*:48-62 (1992).

PTH Receptor: PTH-1 receptor (P1R) is a class IIG protein coupled receptor, the amino-terminal extracellular (N)domain of which is thought to bind with the C-terminal portion of PTH.

PTH Analogs – Structural and Functional Properties

α-aminoisobutyric acid (Aib) and α,α- disubstituted amino acids distinct

from Aib were introduced into short N-terminal PTH peptide analogs. The numerous NMR studies of PTH(1-34) analogs, performed in a variety of polar or non-polar solvents, have generally indicated two domains of secondary structure: a stable C-terminal helix extending approximately from Ser-17 to Val-31, and a shorter and less stable amino-terminal helix, extending variably from Ser-3 to Lys-13, the two domain being connected by a bend or turn region (Marx, U.C., *et al., Biochem. Biophys. Res. Commun. 267*:213-220 (2000); Chen, Z., *et al., Biochemistry 39*:12766-12777 (2000); Marx, U.C., *et al., J. Biol. Chem. 270*:15194-15202 (1995); Marx, U.C., *et al., J. Biol. Chem. 273*:4308-4316 (1998); Pellegrini, M., *et al., Biochemistry 37*:12737-12743 (1998); Gronwald, W., *et al., Biol. Chem. Hoppe Seyler 377*:175-186 (1996); Barden, J.A., and Kemp, B.E., *Biochemistry 32*:7126-7132 (1993)). The recent crystallographic study of PTH(1-34) indicated a continuous α-helix extending from Ser-3 to His-32 and containing only a slight 15° bend at the mid-section. However, NMR data indicates that the N-terminal α-helix is relatively weak. Helix-stabilizing modifications, such as the introduction of Aib residues, offer significant benefits in terms of peptide potency, and result in short peptides (≤14 amino acids) with activity comparable to PTH(1-34).

Described herein are novel "minimized" variants of PTH that are small enough to be deliverable by simple non-injection methods. The variants of the present invention contain substitutions in the first 14 amino acids of the polypeptide. The new polypeptides correspond to the 1-14, 1-13, 1-12, 1-11, 1-10 and 1-9 amino acid sequence of the mature PTH polypeptide, unless otherwise noted. The shorter variants (≤ PTH1-14) have a molecular weight of less than 2,000 daltons.

As protein products, compounds described herein are amenable to production by the techniques of solution- or solid-phase peptide synthesis. The solid phase peptide synthesis technique, in particular, has been successfully applied in the production of human PTH and can be used for the production of these compounds (for guidance, *see* Kimura *et al., supra*, and see Fairwell *et al., Biochem. 22*:2691 (1983)). Success with producing human PTH on a relatively

large scale has been reported by Goud *et al., in J. Bone Min. Res. 6(8)*:781 (1991). The synthetic peptide synthesis approach generally entails the use of automated synthesizers and appropriate resin as solid phase, to which is attached the C-terminal amino acid of the desired compounds of peptides having the sequence of SEQ. ID. NO. 1 or derivatives thereof. Extension of the peptide in the N-terminal direction is then achieved by successively coupling a suitably protected form of the next desired amino acid, using either FMOC- or BOC-based chemical protocols typically, until synthesis is complete. Protecting groups are then cleaved from the peptide, usually simultaneously with cleavage of peptide from the resin, and the peptide is then isolated and purified using conventional techniques, such as by reversed phase HPLC using acetonitrile as solvent and tri-fluoroacetic acid as ion-pairing agent. Such procedures are generally described in numerous publications and reference may be made, for example, to Stewart and Young, "Solid Phase Peptide Synthesis," 2nd Edition, Pierce Chemical Company, Rockford, IL (1984). It will be appreciated that the peptide synthesis approach is required for production of such as for example, a peptide having the sequence of SEQ. ID. NO. 1 and derivatives thereof which incorporate amino acids that are not genetically encoded, such as Aib.

Substituents may be attached to the free amine of the N-terminal amino acid of compounds of the present invention standard methods known in the art. For example, alkyl groups, e.g., C_{1-12} alkyl, are attached using reductive alkylation. Hydroxyalkyl groups, e.g. C_{1-12} hydroxyalkyl, are also attached using reductive alkylation wherein the free hydroxy group is protected with a t-butyl ester. Acyl groups, e.g., COE_1, are attached by coupling the free acid, e.g., E_1COOH, to the free amino of the *N*-terminal amino acid. Additionally, possible chemical modifications of the C-terminal end of the polypeptide are encompassed within the scope of the invention. These modifications may modify binding affinity to the receptor.

Also contemplated within the scope of this invention are those compounds such as for example, a peptide having the sequence of SEQ. ID. NO. 1 and derivatives thereof with altered secondary or tertiary structure, and/or altered

stability, which still retain biological activity. Such derivatives might be achieved through lactam cyclization, disulfide bonds, or other means known to a person of ordinary skill in the art. A preferable embodiment of the invention is drawn to any of the above recited polypeptides, wherein the polypeptide contains a C-terminal amide.

Utility and Administration of Compounds of the Invention

Compounds of the invention or derivatives thereof have multiple uses. These include, *inter alia,* agonists or antagonists of the PTH receptor, prevention and treatment of a variety of mammalian conditions manifested by loss of bone mass, diagnostic probes, antigens to prepare antibodies for use as diagnostic probes and even as molecular weight markers. Being able to specifically substitute one or more amino acids in the PTH polypeptide permits construction of specific molecular weight polypeptides.

In particular, the compounds of this invention are indicated for the prophylaxis and therapeutic treatment of osteoporosis and osteopenia in humans. Furthermore, the compounds of this invention are indicated for the prophylaxis and therapeutic treatment of other bone diseases. The compounds of this invention are also indicated for the prophylaxis and therapeutic treatment of hypoparathyroidism. Finally, the compounds of this invention are indicated for use as agonists for fracture repair and as antagonists for hypercalcemia.

In general, compounds of the present invention, or salts thereof, are administered in amounts between about 0.01 and 1 μg/kg body weight per day, preferably from about 0.07 to about 0.2 μg/kg body weight per day. For a 50 kg human female subject, the daily dose of biologically active compound is from about 0.5 to about 50 μgs, preferably from about 3.5 to about 10 μgs. In other mammals, such as horses, dogs, and cattle, higher doses may be required. This dosage may be delivered in a conventional pharmaceutical composition by a single administration, by multiple applications, or via controlled release, as needed to achieve the most effective results, preferably one or more times daily by injection.

For example, this dosage may be delivered in a conventional pharmaceutical composition by nasal insufflation or by orally active means. Another method of administration includes subcutaneous injection, at least once daily, for potential anabolic effects on the skeleton.

The selection of the exact dose and composition and the most appropriate delivery regimen will be influenced by, *inter alia*, the pharmacological properties of the selected compounds of the invention, the nature and severity of the condition being treated, and the physical condition and mental acuity of the recipient.

Representative preferred delivery regimens include, without limitation, oral, parenteral, subcutaneous, transcutaneous, intramuscular and intravenous, rectal, buccal (including sublingual), transdermal, and intranasal insufflation.

Pharmaceutically acceptable salts retain the desired biological activity of the compounds of the invention without toxic side effects. Examples of such salts are (a) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; and salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, pamoic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acids, naphthalene disulfonic acids, polygalacturonic acid and the like; (b) base addition salts formed with polyvalent metal cations such as zinc, calcium, bismuth, barium, magnesium, aluminum, copper, cobalt, nickel, cadmium, and the like; or with an organic cation formed from N,N′-dibenzylethylenediamine or ethylenediamine; or (c) combinations of (a) and (b), e.g., a zinc tannate salt and the like. Pharmaceutically acceptable buffers include but are not limited to saline or phosphate buffered saline. Also included in these solutions may be acceptable preservative known to those of skill in the art.

A further aspect of the present invention relates to pharmaceutical compositions comprising as an active ingredient compounds of the invention or derivatives thereof of the present invention, or pharmaceutically acceptable salt

thereof, in admixture with a pharmaceutically acceptable, non-toxic carrier. As mentioned above, such compositions may be prepared for parenteral (subcutaneous, transcutaneous, intramuscular or intravenous) administration, particularly in the form of liquid solutions or suspensions; for oral or buccal administration, particularly in the form of tablets or capsules; for rectal, transdermal administration; and for intranasal administration, particularly in the form of powders, nasal drops or aerosols.

The compositions may conveniently be administered in unit dosage form and may be prepared by any of the methods well-known in the pharmaceutical art, for example as described in Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Company, Easton, Pa., (1985), incorporated herein by reference. Formulations for parenteral administration may contain as excipients sterile water or saline, alkylene glycols such as propylene glycol, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes and the like. For oral administration, the formulation can be enhanced by the addition of bile salts or acylcarnitines. Formulations for nasal administration may be solid and may contain excipients, for example, lactose or dextran, or may be aqueous or oily solutions for use in the form of nasal drops or metered spray. For buccal administration typical excipients include sugars, calcium stearate, magnesium stearate, pregelatinated starch, and the like.

When formulated for one preferred route of administration, nasal administration, the absorption across the nasal mucous membrane may be enhanced by surfactant acids, such as for example, glycocholic acid, cholic acid, taurocholic acid, ethocholic acid, deoxycholic acid, chenodeoxycholic acid, dehydrocholic acid, glycodeoxycholic acid, cyclodextrins and the like in an amount in the range between about 0.2 and 15 weight percent, preferably between about 0.5 and 4 weight percent, most preferably about 2 weight percent.

Delivery of the compounds of the present invention to the subject over prolonged periods of time, for example, for periods of one week to one year, may be accomplished by a single administration of a controlled release system containing sufficient active ingredient for the desired release period. Various

controlled release systems, such as monolithic or reservoir-type microcapsules, depot implants, osmotic pumps, vesicles, micelles, liposomes, transdermal patches, iontophoretic devices and alternative injectable dosage forms may be utilized for this purpose. Localization at the site to which delivery of the active ingredient is desired is an additional feature of some controlled release devices, which may prove beneficial in the treatment of certain disorders.

One form of controlled release formulation contains the polypeptide or its salt dispersed or encapsulated in a slowly degrading, non-toxic, non-antigenic polymer such as copoly(lactic/glycolic) acid, as described in the pioneering work of Kent, Lewis, Sanders, and Tice, U.S. Pat. No. 4,675,189. The compounds or, preferably, their relatively insoluble salts, may also be formulated in cholesterol or other lipid matrix pellets, or silastomer matrix implants. Additional slow release, depot implant or injectable formulations will be apparent to the skilled artisan. See, for example, Sustained and Controlled Release Drug Delivery Systems, J. R. Robinson ed., Marcel Dekker, Inc., New York, 1978, and R. W. Baker, Controlled Release of Biologically Active Agents, John Wiley & Sons, New York, 1987.

Like PTH, the PTH variants may be administered in combination with other agents useful in treating a given clinical condition. When treating osteoporosis and other bone-related disorders for example, the PTH variants may be administered in conjunction with a dietary calcium supplement or with a vitamin D analog (*see* U.S. Pat. No. 4,698,328). Alternatively, the PTH variant may be administered, preferably using a cyclic therapeutic regimen, in combination with bisphosphonates, as described for example in U.S. Pat. No. 4,761,406, or in combination with one or more bone therapeutic agents such as, without limitation, calcitonin and estrogen.

PTH Analog Receptor-Signaling Activities

A crucial step in the expression of hormonal action is the interaction of hormones with receptors on the plasma membrane surface of target cells. The formation of hormone-receptor complexes allows the transduction of extracellular

signals into the cell to elicit a variety of biological responses.

Polypeptides described herein can be screened for their agonistic or antagonistic properties using the cAMP accumulation assay. Cells expressing PTH-1 receptor on the cell surface are incubated with native PTH(1-84) (SEQ. ID. NO. 18) for 5-60 minutes at 37°C., in the presence of 2 mM IBMX (3-isobutyl-1-methyl-xanthine, Sigma, St. Louis, MO). Cyclic AMP accumulation is measured by specific radio-immunoassay. A compound that competes with native PTH(1-84) or PTH(1-34) (SEQ. ID. NO.19) for binding to the PTH-1 receptor, and that inhibits the effect of native PTH(1-84) or PTH(1-34) on cAMP accumulation, is considered a competitive antagonist. Such a compound would be useful for treating hypercalcemia.

Conversely, a PTH analog described herein or a derivative thereof that does not compete with native PTH(1-84) or PTH(1-34) for binding to the PTH-1 receptor, but which still prevents native PTH(1-84) or PTH(1-34) activation of cAMP accumulation (presumably by blocking the receptor activation site) is considered a non-competitive antagonist. Such a compound would also be useful for treating hypercalcemia.

The compounds described herein that compete with native PTH(1-84) or PTH(1-34)) for binding to the PTH-1 receptor, and which stimulates cAMP accumulation in the presence or absence of native PTH(1-84) or PTH(1-34) are competitive agonists. A compound that does not compete with native PTH(1-84) or PTH(1-34) for binding to the PTH-1 receptor but which is still capable of stimulating cAMP accumulation in the presence or absence of native PTH(1-84) or PTH(1-34), or which stimulates a higher cAMP accumulation than that observed by a compound of the invention or a derivative thereof alone, would be considered a non-competitive agonist.

Therapeutic Uses of PTH Analogs

Some forms of hypercalcemia and hypocalcemia are related to the interaction between PTH and PTHrP and the PTH-1 and receptors. Hypercalcemia

is a condition in which there is an abnormal elevation in serum calcium level; it is often associated with other diseases, including hyperparathyroidism, osteoporosis, carcinomas of the breast, lung and prostate, epidermoid cancers of the head and neck and of the esophagus, multiple myeloma, and hypernephroma. Hypocalcemia, a condition in which the serum calcium level is abnormally low, may result from a deficiency of effective PTH, *e.g.*, following thyroid surgery.

By "agonist" is intended a ligand capable of enhancing or potentiating a cellular response mediated by the PTH-1 receptor. By "antagonist" is intended a ligand capable of inhibiting a cellular response mediated by the PTH-1 receptor. Whether any candidate "agonist" or "antagonist" of the present invention can enhance or inhibit such a cellular response can be determined using art-known protein ligand/receptor cellular response or binding assays, including those described elsewhere in this application.

In accordance with yet a further aspect of the invention, there is provided a method for treating a medical disorder that results from altered or excessive action of the PTH-1 receptor, comprising administering to a patient therapeutically effective amount of a compound of the invention or a derivative thereof sufficient to inhibit activation of the PTH-1 receptor of said patient.

In this embodiment, a patient who is suspected of having a disorder resulting from altered action of the PTH-1 receptor can be treated using compounds of the invention or derivatives thereof of the invention which are a selective antagonists of the PTH-1 receptor. Such antagonists include compounds of the invention or derivatives thereof of the invention which have been determined (by the assays described herein) to interfere with PTH-1 receptor-mediated cell activation or other derivatives having similar properties.

To administer the antagonist, the appropriate compound of the invention or a derivative thereof is used in the manufacture of a medicament, generally by being formulated in an appropriate carrier or excipient such as, *e.g.*, physiological saline, and preferably administered intravenously, intramuscularly, subcutaneously, orally, or intranasally, at a dosage that provides adequate inhibition of a compound of the invention or a derivative thereof binding to the

PTH-1 receptor. Typical dosage would be 1 ng to 10 mg of the peptide per kg body weight per day.

In accordance with yet a further aspect of the invention, there is provided a method for treating osteoporosis, comprising administering to a patient a therapeutically effective amount of a compound of the invention or a derivative thereof, sufficient to activate the PTH-1 receptor of said patient. Similar dosages and administration as described above for the PTH/PTHrP antagonist, can be used for administration of a PTH/PTHrP agonist, *e.g.*, for treatment of conditions such as osteoporosis, other metabolic bone disorders, and hypoparathyroidism and related disorders.

It will be appreciated by those skilled in the art that the invention can be performed within a wide range of equivalent parameters of composition, concentration, modes of administration, and conditions without departing from the spirit or scope of the invention or any embodiment thereof.

Having now fully described the invention, the same will be more readily understood by reference to specific examples which are provided by way of illustration, and are not intended to be limiting of the invention, unless herein specified.

Examples

The following protocols and experimental details are referenced in the examples that follow.

Example 1

Materials and Methods

Peptides. The amino acid sequences of the peptides used in the study were all derived from human or rat PTH sequences and contain a free amino-terminus and an amidated C-terminus. The parent peptide used as a starting scaffold was [M]PTH(1-14), which is defined as [$Ala^{1,3,12}$,Gln^{10},Har^{11}, Trp^{14}]PTH(1-14)NH_2 (SEQ. ID. NO. 13). Peptides were prepared on automated peptide synthesizers

(model 430A PE, Applied Biosystems, Foster City, CA, or Model 396 MBS Advanced Chem Tect, Louisville, KY) using FMOC main-chain protecting group chemistry, HBTU/HOBt/DIEA (1:1:2 molar ratio) for coupling reactions, and TFA-mediated cleavage/sidechain-deprotection (MGH Biopolymer Synthesis Facility, Boston, MA). All peptides were desalted by adsorption on a C18-containing cartridge, and purified further by HPLC. The dry peptide powders were reconstituted in 10 mM acetic acid and stored at −80°C. The purity, identity, and stock concentration for each peptide was secured by analytical HPLC, Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry and amino acid analysis. Radiolabeling of [M]PTH(1-21) (SEQ.ID.NO. 20) and [$Aib^{1,3}$,M]PTH(1-21) (SEQ.ID.NO.21) was performed using ^{125}I-Na (2,200 Ci/mmol, NEN) and chloramine-T; the resultant radioligands were purified by HPLC.

Cell Culture. The cell line HKRK-B28 (Takasu, H., *et al., J. Bone Miner. Res. 14*:11-20 (1999)) was derived from the porcine kidney cell line, LLC-PK_1 by stable transfection with plasmid DNA encoding a recombinant P1R chimera comprised of the opposum P1R from the N-terminus to the mid region of TM3 and the rat P1R from the mid region of TM3 to the C-terminus. The surface density of the P1R in these cells is ~280,000 receptors per cell. The clonal cell line LdelNt-2 was derived from LLC-PK_1 cells via stable transfection with a plasmid encoding P1R-delNt, a recombinant human PTH-1 receptor construct in which most of the amino-terminal extracellular domain is deleted. These cells, as well as COS-7 cells and SaOS-2-B10 cells, were cultured at 27°C in T-75 flasks (75 mm^2) in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (10%), penicillin G (20 units/ml), streptomycin sulfate (20 μg/ml) and amphotericin B (0.05 μg/ml) in a humidified atmosphere containing 5% CO_2. Stock solutions of EGTA/trypsin and antibiotics were from GIBCO; fetal bovine serum was from Hyclone Laboratories (Logan, UT). COS-7 cells sub-cultured in 24-well plates were transfected with plasmid DNA (200 ng per well) encoding the wild-type human P1R or truncated human P1R deleted for residues (24-181) (Shimizu, M., *et al., J. Biol. Chem. 275*:21836-21843 (2000)) that was purified by cesium chloride/ethidium bromide density gradient centrifugation, and FuGENE 6

transfection reagent (Roche Indianapolis IN) according to the manufacturer's recommended procedure. All cells, in 24-well plates, were treated with fresh media and shifted to 33 °C for 12 to 24 h prior to assay.

cAMP Stimulation. Stimulation of cells with peptide analogs was performed in 24-well plates. Cells were rinsed with 0.5 mL of binding buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 5% heat-inactivated horse serum, 0.5% fetal bovine serum, adjusted to pH 7.5 with HCl) and treated with 200 μL of cAMP assay buffer (Delbecco's modified Eagle's medium containing 2 mM 3-isobutyl-1-methylxanthine, 1 mg/mL bovine serum albumin, 35 mM Hepes-NaOH, pH 7.4) and 100 μL of binding buffer containing varying amounts of peptide analog (final volume = 300μL). The medium was removed after incubation for 30 to 60 minutes at room temperature, and the cells were frozen on dry ice, lysed with 0.5 mL 50 mM HCl, and refrozen (~80 °C). The cAMP content of the diluted lysate was determined by radioimmunoassay. The EC_{50} response values were calculated using nonlinear regression (see below).

FMOC. (Fluorenylmethoxycarbonyl group) A group used for linkage to amino groups for the purpose either of forming fluorescent amino-acid derivatives that can readily be detected after column chromatography, or to protect the amino groups of amino acids or nucleotides while other functional groups are undergoing reaction. Reagents useful for introducing the group are 9-fluorenylmethyl chloroformate and 9-fluorenyl-methyl succinimidyl carbonate.

Competition Binding. Binding reactions were performed with HKRK-B28 cells or in COS-7 cells in 24-well plates. The cells were rinsed with 0.5 mL of binding buffer, and then treated successively with 100 μL binding buffer, 100 μL of binding buffer containing various amounts of unlabeled competitor ligand, and 100 μL of binding buffer containing ca. 100,000 cpm of ^{125}I-[$Aib^{1,3}$,Nle^{8},Gln^{10},Har^{11},Ala^{12},Trp^{14}, Arg^{19},Tyr^{21}]rPTH(1-21) (SEQ. ID. NO. 36) (ca. 26 fmol; final volume = 300 μL). Incubations were 4 to 6 h at 4 °C, at which time near equilibrium conditions were attained. Cells were then placed on ice, the binding medium was removed, and the monolayer was rinsed three times with 0.5 mL of cold binding buffer. The cells were subsequently lysed with 0.5 mL 5N

NaOH and counted for radioactivity. For each tracer and in each experiment, the non-specific binding was determined as the radioactivity that bound in the presence of the same unlabeled peptide at a concentration of 1 μM, and was ~1% of total radioactivity added for each tracer. The maximum specific binding (B_0) was the total radioactivity bound in the absence of competing ligand, corrected for nonspecific binding, and for each tracer, ranged from 8% to 20% of the total radioactivity added. Nonlinear regression was used to calculate binding IC_{50} values (see below). Scatchard transformations of homologous competition binding data derived from studies with 26 fmol of ^{125}I-[$Aib^{1,3}$,Nle^8,Gln^{10},Har^{11},Ala^{12},Trp^{14}, Arg^{19},Tyr^{21}]rPTH(1-21) (SEQ. ID. NO. 36) were employed for estimations of apparent equilibrium dissociation constant (k_{Dapp}s) and total number of ligand binding sites (B_{max}), assuming a single class of binding sites and equal affinities of the iodinated and non iodinated ligand.

Stimulation of Inositol Phosphate Production. COS-7 cells transfected as above with P1R-WT were treated with serum-free, inositol-free DMEM containing 0.1% bovine serum albumin and [^{3}H]myo-inositol (NEN, Boston, MA) (2μCi/mL) for 16 h prior to assay. At the time of the assay, the cells were rinsed with binding buffer containing LiCl (30 mM) and treated with the same buffer with or without a PTH analog. The cells were then incubated at 37°C for 40 min, after which the buffer was removed and replaced by 0.5 mL of ice cold 5% trichloroacetic acid solution. After 3 h on ice, the lysate was collected and extracted twice with ethyl ether. The lysate was then applied to an ion exchange column (0.5 mL resin bed) and the total inositol phosphates were eluted as described previously (Berridge, M.J., *et al., Biochem. J. 212*:473-482 (1983)), and counted in liquid scintillation cocktail.

Inhibition of Chondrocyte Differentiation in Embryonic Mouse Metatarsals. Metatarsals from embryonic day (E) 15.5 mouse embryos were excised and cultured in a 37°C humidified incubator (5% CO_2) in serum-free αMEM media in 24 well plates. Sixteen hours later, a PTH analog or vehicle was added, and the samples were incubated for an additional 48 h in 37°C with peptide or vehicle added again at the 24 h time point. At the end of the 64 h incubation

period, the samples were fixed with 10% formalin/phosphate-buffered saline, then directly visualized on a dissecting microscope using white light. Sections were processed for in-situ hybridization analysis using ^{35}S-labeled riboprobes specific for collagen type X mRNA, a developmental marker gene expressed only in hypertrophic chrondrocytes of the growth plate.

Circular Dichroism. Circular Dichroism spectra were recorded on a Jasco model 710 spectropolarimeter; peptides were analyzed at a concentrtion of 20 μM in 50 mM sodium phosphate buffer pH 7.4, or the same buffer containing 2,2,2-trifluoroethanol at 20% (v/v). Spectroscopic scans were performed at 20°C and at wavelengths between 185 and 255 nM, with data recored at each 1 nM interval. The spectral bandwidth was 1.5 nM and 8 scans were accumulated and averaged for each sample. At each wavelength, the mean residue elipticity [θ x 100/l x C x n); where θ is the raw elipticity value (in dimensions of millidegree), l is the sample path length, C = is the molar peptide concentration, and n is the number of residues in the peptide (Bowen, W.P., and Jerman, J.C., *Trends in Pharmacol. Sci. 16*: 413-417 (1995)). The helical content of each peptide was estimated by dividing [θ] observed at 222 nM for that peptide by -28,100, which is the reported $[\theta]_{222}$obs for a model helical decapeptide (Bowen, W.P., and Jerman, J.C., *Trends in Pharmacol. Sci. 16*: 413-417 (1995)).

Data Calculation. Calculations were performed using Microsoft® Excel. Nonlinear regression analyses of binding and cAMP dose-response data were performed using the four-parameter equation: y_p = Min + [(Max - Min)/(1 + $(IC_{50}/x)^{slope}$)]. The Excel Solver function was utilized for parameter optimization, as described previously (Carter, P.H., *et al., Endocrinology 140*: 4972-4981 (1999); Bowen, W.P., and Jerman, J.C., *Trends in Pharmacol. Sci. 16*: 413-417 (1995)). Differences between paired data sets were statistically evaluated using a one-tailed Student's t-test, assuming unequal variances for the two sets.

Example 2

P1-R Binding Affinity of Analogs Containing α,α-disubstituted Amino Acids

The effects of introducing α,α-disubstituted amino acids distinct from Aib at positions 1 and/or 3 of [M]PTH(1-14)(M=$Ala^{1,3,12}$, Gln^{10}, Har^{11},Trp^{14}) (SEQ. ID. NO. 13) were analyzed. Six amino acids were chosen: α-Amino-isobutyric acid (Aib), α,α-diethylglycine (Deg); 1-aminocyclopropane-1-carboxylic acid (Ac_3c); 1-amino-cyclobutane-carboxylic acid (Ac_4c), 1-aminocyclopentane-1-carboxylic acid (Ac_5c), and 1-amino-cyclohexane-carboxylic acid (Ac_6c) (some of which are shown in Fig. 1).

In competition binding assays performed in an LLC-PK1-derived cell line (B28) that stably expresses the hPTH receptor using ^{125}I-[$Aib^{1,3}$,Nle^8,Gln^{10},Har^{11},Ala^{12},Trp^{14}, Arg^{19},Tyr^{21}]rPTH(1-21) (SEQ. ID. NO. 36) tracer, the affinities of the Deg^3- and Ac_5c^3-containing analog were comparable to that of [M]PTH(1-14)(IC_{50}s~3μM). The affinity of Ac_3c^3-containing analog was 14-fold lower than that of [M]PTH(1-14), and the affinities of the Deg-, Ac_3c- and Ac_5c-substituted peptides were 3-50 fold higher than that of [M]PTH(1-14)(IC_{50}=0.6μM).

The analog [Ac_5c^1,Aib^3,M]PTH(1-14) (SEQ. ID. NO. 15) exhibited one of the highest binding affinities (IC_{50}=100 nM) and one of the highest cAMP potencies {EC_{50}=0.9nM, compared to 200nM for [M]PTH(1-14)} of any PTH(1-14) analogs studied to date. The Ac_5c^1 modification improved affinity 50-fold relative to the parent [M]PTH(1-14) analog {[$Ala^{1,3,12}$,Gln^{10},Har^{11}, Trp^{14}]humanPTH(1-14)-NH_2}(SEQ. ID. NO. 13), and it improved cAMP potency ~35 fold. A cyclopentane ring at position -1 therefore enables a more favorable interaction with the P1R than do the two C^α-methyl of Aib. That each of these structurally distinct amino acids at position 1 improves affinity/potency suggests that their enhancing effects are not due to their specific side chain topologies, but rather to their effects on backbone conformation, that is stabilization of α-helix structure. Deg^3 substitution showed marginal improvement of affinity by around 1.5 fold, but markedly diminished signaling potency by ~40 fold.

Example 3

Ac_5c Peptide Agonist Activity

The peptide [Ac_5c^1, Aib^3, M]PTH(1-14)(SEQ. ID. NO. 15) was ~ 2-fold more potent than [$Aib^{1,3}$,M]PTH(1-14) (SEQ. ID. NO. 3) for inhibiting ^{125}I-[$Aib^{1,3}$,Nle^8,Gln^{10},Har^{11},Ala^{12},Trp^{14}, Arg^{19},Tyr^{21}]rPTH(1-21) (SEQ. ID. NO. 36) binding to B28 cells, and for stimulating adenylyl cyclase, as well as for stimulating phospholipase C (Fig. 2). This peptide is thus one of the most potent PTH(1-14) analog identified so far. Combining Deg^1 and Deg^3 yielded a peptide that bound with adequate affinity (Fig. 1A) but was a true partial agonist for cAMP formation (Fig. 1B).

The peptide [Ac_5c^1, Aib^3, Gln^{10}]PTH(1-10) (SEQ. ID. NO. 22) was a full cAMP agonist, albeit with micromolar potency at 10^{-4}M, [Ac_5c^1, Aib^3]PTH (1-9) (SEQ. ID. NO. 31) exhibited clear agonist activity making it the shortest PTH analog peptide with reliable cAMP agonist activity.

Example 4

Ac_5c Peptide Antagonist Activity

Ligands that function as antagonists can be useful for treating hyperparathyroidism. Analogs of [Ac_5c^1, Aib^3, M]PTH(1-14)(parent) (SEQ. ID. NO. 15) containing N-terminal modifications predicted to dissociate signaling and binding affinity (e.g. desamino-1, Trp-2, Bpa-2, Arg-2, Deg-1,3). Most of the substitutions moderately reduced P1R binding affinity and strongly reduced cAMP-stimulating potency.

The Trp^2 analog [Ac_5c^1,Trp^2,Aib^3, M]PTH(1-14) (SEQ. ID. NO. 23) at 10^{-5}M inhibits the agonist activity of [$Aib^{1,3}$, M]PTH(1-14) (SEQ. ID. NO. 3) at 10^{-9}M by ~50%.

Example 5

Single Substitutions at Positions 1 and 3 in PTH (1-14)

Single substitutions of di-alkyl amino acids were introduced at positions 1 and 3 in the parent scaffold peptide [$Ala^{1,3,12}$,Gln^{10},Har^{11},Trp^{14}]PTH(1-14)NH_2 (SEQ.ID.NO.13). Schematic structures of the amino acids utilized at positions 1 and 3 in these studies are shown in Figure 1, and the peptide sequences are presented in Table 1. The parent peptide [$Ala^{1,3,12}$,Gln^{10},Har^{11},Trp^{14}]PTH(1-14)NH_2 (SEQ. ID. NO. 13), which contains alanine at positions 1 and 3, stimulated cAMP accumulation in HKRK-B28 cells with a potency (EC_{50}) value of 220±80nM, and it inhibited the binding of ^{125}I-[$Aib^{1,3}$,Nle^{8},Gln^{10},Har^{11},Ala^{12},Trp^{14}, Arg^{19},Tyr^{21}]rPTH(1-21) (SEQ. ID. NO. 36) tracer radioligand to these cells with an apparent affinity (IC_{50}) value of 27±3μM. Relative to the parent analog, the analogs substituted at position 1 were ~two-fold (Ac_3c, P=0.1), 11-fold (Deg, P=0.02) or 61-fold (Ac5c, P=0.02) more potent for stimulating cAMP accumulation, and these increases in potency were accompanied by commensurate effects on apparent binding affinity. At position 3, substitution with either cycloalkane amino acid, Ac_3c or Ac_5c, increased cAMP-stimulating potency modestly (<two-fold), whereas substitution with the linear amino acid, Deg, diminished potency approximately 10-fold; Ac_5c-3 and Deg-3 had little or no effect on binding affinity, whereas Ac_3c-3 reduced affinity approximately 10-fold.

Example 6

Combined Substitutions at Positions 1 and 3 in PTH(1-14) analogs

Di-alkyl amino acids were introduced at both positions 1 and 3 of the parent scaffold peptide [$Ala^{1,3,12}$,Gln^{10},Har^{11},Trp^{14}]PTH(1-14)NH_2 (SEQ.ID.NO.13). Introducing Ac_3c at positions 1 and 3 yielded the analog [$Ac_3c^{1,3}$,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2 (SEQ. ID. NO. 28), which was comparable to the parent peptide in terms of binding affinity and signaling potency. Introducing Ac_5c at these positions yielded the analog [$Ac_5c^{1,3}$,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2 (SEQ. ID. NO. 29), which bound to the P1R with 20-fold higher affinity than did the parent peptide (P=0.001) and was 30-fold more potent for cAMP signaling (P=0.02; Table 2). Combining the

Deg substitutions at positions 1 and 3 yielded [$Deg^{1,3}$,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2 (SEQ. ID. NO. 27), which bound to the P1R with an affinity 50-fold higher than that of the parent (*P*=0.001) but elicited only a partial agonist response which attained a maximum that was only 45% of that attained by the parent peptide.

Example 7
PTH(1-9) and PTH(1-10) Analogs

Minimum-length active analogs were developed to be used to functionally probe the PTH•PTH receptor interaction mechanism. Native N-terminal PTH analogs that are shorter than PTH(1-14) are inactive in cell-based assays, measurable cAMP responses can be detected in P1R-transfected cells for the analog [$Aib^{1,3}$,Gln^{10}]PTH(1-10)NH_2(SEQ. ID. NO.35). [Ac_5c^1,Aib^3,Gln^{10}]PTH(1-10)NH_2(SEQ. ID. NO. 22) and [Ac_5c^1,Aib^3]PTH(1-9)NH_2 (SEQ. ID. NO. 31) were prepared and their activity was assessed in HKRK-B28 cells. When tested at concentrations as high as $1x10^{-4}$M, neither peptide inhibited the binding of P(1-21) tracer radioligand. Each analog induced a clear cAMP response in these cells. From dose-response analysis of [Ac_5c^1,Aib^3,Gln^{10}]PTH(1-10)NH_2 (SEQ. ID. NO. 22), EC_{50} value was estimated at approximately 3 μM. The PTH(1-19) analog was assayed at a single high concentration ($1x10^{-4}$M) and found to induce a six-fold increase in cAMP levels, relative to the basal levels (*P*=0.001); at the same concentrations, [$Aib^{1,3}$,Gln^{10}]PTH(1-10)NH_2 (SEQ. ID. NO. 35) induced a two-fold increase in cAMP accumulation (*P*=0.52) and native PTH(1-9) (SEQ. ID. NO. 33) was inactive.

Example 8
Analog Activity in P1R-delNT cells

Agonist activities of selected analogs in cells expressing P1R-delNt were examined. This PTH-1 receptor construct lacks most of the N domain, but nevertheless mediates full agonist responses to N-terminally modified PTH

analogs, and thus enables the relative roles of the receptor's N and J domains to the ligand interaction process to be evaluated. The clonal cell line LdelNT-2 was used, which was derived from LLC-PK_1 cells by stable transfection with a plasmid encoding the human P1R-delNt construct. In these cells, [Ac_5c^1, Aib^3,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2 (SEQ. ID. NO.15) induced a 70-fold increase in cAMP accumulation with high potency (EC_{50}=6.4±2.3nM); [Ac_5c^1,Aib^3,Gln^{10}]PTH(1-10)NH_2 (SEQ. ID. NO. 22) exhibited weaker potency (EC_{50}~40μM) and [$Deg^{1,3}$,Gln^{10},Har^{11},Ala^{12},Trp^{14}]PTH(1-14)NH_2 (SEQ. ID. NO. 27) exhibited partial agonist behavior (E_{max}=23-fold over basal; EC_{50} =1.4±0.5μM).

Table 1: PTH Peptide Sequences

	1 5 10 14
PTH(1-14)NH$_2$	Ser-V-Ser-E-I-Q-L-M-H-N-Leu-G-K-H(SEQ. ID. NO. 26)
[M]PTH(1-14) (Parent)	A-V-A-E-I-Q-L-M-H-Q-Har-A-K-W (SEQ. ID. NO. 13)
[Deg^1,M]PTH(1-14)	Deg-V-A-E-I-Q-L-M-H-Q-Har-A-K-W(SEQ. ID. NO.24)
[Ac$_3$c^1,M]PTH(1-14)	Ac$_3$c-V-A-E-I-Q-L-M-H-Q-Har-A-K-W(SEQ. ID. NO. 25)
[Ac$_5$c^1,M]PTH(1-14)	Ac$_5$c-V-A-E-I-Q-L-M-H-Q-Har-A-K-W(SEQ. ID. NO. 4)
[Deg^{1,3},M]PTH(1-14)	Deg-V-Deg-E-I-Q-L-M-H-Q-Har-A-K-W(SEQ. ID. NO. 27)
[Ac$_3$c^{1,3},M]PTH(1-14)	Ac$_3$c-V-Ac$_3$c-E-I-Q-L-M-H-Q-Har-A-K-W (SEQ. ID. NO. 28)
[Ac$_5$c^{1,3},M]PTH(1-14)	Ac$_5$c-V-Ac$_5$c-E-I-Q-L-M-H-Q-Har-A-K-W (SEQ. ID. NO. 29)
[Aib^{1,3},M]PTH(1-14)	Aib-V-Aib-E-I-Q-L-M-H-Q-Har-A-K-W (SEQ.ID. NO.3)
[Ac$_5$c^1,Aib^3,M]PTH(1-14)	Ac$_5$c-V-Aib-E-I-Q-L-M-H-Q-Har-A-K-W (SEQ.ID. NO. 15)
[Ac$_5$c^1,Aib^3,M]PTH(1-10)	Ac$_5$c-V-Aib-E-I-Q-L-M-H-Q (SEQ. ID. NO. 22)
[Ac$_5$c^1,Aib^3,M]PTH(1-9)	Ac$_5$c-V-Aib-E-I-Q-L-M-H (SEQ. ID. NO. 31)
[Ac$_4$c^1, Aib^3,M]PTH(1-14)	Ac$_4$c-V-Aib-E-I-Q-L-M-H-Q-Har-A-K-W (SEQ. ID. NO. 7)
[Ac$_6$c^1, Aib^3,M]PTH(1-14)	Ac$_6$c-V-Aib-E-I-Q-L-M-H-Q-Har-A-K-W (SEQ. ID. NO. 8)
[Ac$_5$c^1, Ac$_4$c^3,M]PTH(1-14)	Ac$_5$c-V-Ac$_4$c-E-I-Q-L-M-H-Q-Har-A-K-W (SEQ. ID. NO.9)
[Ac$_5$c^1, Ac$_6$c^3,M]PTH(1-14)	Ac$_5$c-V-Ac$_6$c-E-I-Q-L-M-H-Q-Har-A-K-W (SEQ. ID. NO. 10)
[Ac$_4$c^1, Ac$_4$c^3,M]PTH(1-14)	Ac$_4$c-V-Ac$_4$c-E-I-Q-L-M-H-Q-Har-A-K-W (SEQ. ID. NO. 11)
[Ac$_6$c^1, Ac$_3$c^3,M]PTH(1-14)	Ac$_6$c-V-Ac$_6$c-E-I-Q-L-M-H-Q-Har-A-K-W (SEQ. ID. NO. 12)
Tracer Radioligands: ^{125}I-[M*]PTH(1-21)	Aib-V-Aib-E-I-Q-L-Nle-H-Q-Har-A-K-W-L-A-S-V-R-R-Y* (SEQ. ID. NO. 36)

* indicates the iodinated tyrosine

Having now fully described the present invention in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious to one of ordinary skill in the art that the same can be performed by modifying or changing the invention with a wide and equivalent range of conditions, formulations and other parameters thereof, and that such modifications or changes are intended to be encompassed within the scope of the appended claims.

All publications, patents and patent applications mentioned herein above are herein incorporated in their entirety and by reference to the same extent as if

each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.